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(54) Title: THE PRODUCTION OF HUMAN GRANULOCYTE COLONY-STIMULATING FACTOR

(57) Abstract

The present invention relates to the production of human granulocyte colony-stimulating factor (hG-CSF). A recombinant plasmid (pYHM-G-CSF) containing cDNA for hG-CSF was produced and introduced into Escherichia coli (E. coli) which was developed to recombinant bacteria. The recombinant bacteria have high expression ability to 1.7g hG-CSF from 1L culture media. The refolded and purified hG-CSF has comparable biological activity of G-CSF.

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THE PRODUCTION OF HUMAN GRANULOCYTE COLONY-STIMULATING FACTOR

FIELD OF THE INVENTION

The present invention relates to the production of human granulocyte colony-stimulating factor(hG-CSF). A recombinant plasmid (pYHM-G-CSF) containing cDNA for hG-CSF was produced and introduced into Escherichia coli (E. coli) which was developed to recombinant bacteria. The recombinant bacteria have high expression ability to 1.7g hG-CSF from 1L culture media. The refolded and purified hG-CSF has comparable biological activity of G-CSF.

DESCRIPTION OF THE PRIOR ART

Granulocytes or monocytes were found in the part of upper layer of a culture system, which is consisted of lower layer of human kidney or fetal cells and upper layer of human bone marrow cells. This finding suggested the existence of the colony-stimulating factor (CSF) [Pluznik and Sach, J. Cell. Comp. Physiol. 66:319 (1965); Bradley and Metcalf, Aust. J. Exp. Biol. Med. Sci. 44:287 (1966)]. The factors, called CSFs, are produced from normal cells, such as T-cells, monocytes and myeloid cells. CSFs effect on stem cells of granulocytes or monocytes. There are several CSFs, such as the granulocyte-macrophage colony

stimulating factors (GM-CSFs), macrophage colony stimulating factors (M-CSFs), multi-colony stimulating factors (multi-CSFs), and granulocyte colony stimulating factors (G-CSFs). These factors function on different target cells and release on the different differentiation stages of the cells.

It was reported that G-CSFs were produced from human normal tissues or myeloma cells. hG-CSF from human myeloma cells is composed of 177 amino acid sequence and 536 bp nucleotides of the gene (US patent No. 4,833,127).

hG-CSF is used for the treatment of hematopoiesis disorder from cancer chemotherapy, cancer irradiation therapy, or other problems (US patent No. 5,186,931; US patent No. 5,202,117; WO 92-11022; WO 92-14480).

hG-CSF has been attemped to be mass-produced for the above treatment. The mass production of hG-CSF was possible, but it was expressed as a fusion protein, which should be further processed, such as the cutting of the fusion protein and purification, resulting in the low yield.

Many methods have been used as the purification from human specimen, the separation from human cell culture, the production from hybridoma by cell-fusion method with hG-CSF producing cells, the production from E. coli or animal cells by recombinant techniques, hG-CSF analogs with

different amino acid sequences. The problems of the above methods were low yield by many purification steps, resulting in economical problems.

OBJECTS OF THE INVENTION

The purpose of this invention is to provide the high yield of highly purified human granulocyte colony-stimulating factor (hG-CSF).

The purpose of this invention is to provide the production method of hG-CSF.

The purpose of this invention is to provide the recombinant plasmin including cDNA of hG-CSF.

BRIEF DESCRIPTION OF THE DRAWINGS

The objects and features of the present invention will become apparent from the following description given in conjunction with the accompanying drawing, in which:

Figure 1 shows the DNA primers employed to synthesize cDNA for hG-CSF.

Figure 2 represents the construction of recombinant plasmid, pYHM-G-CSF, containing cDNA for hG-CSF and an additional peptide (pelB).

Figure 3 represents the results of 12% SDS-PAGE (panel A) and western blot (panel B) of hG-CSF.

Lane 1: The cell lysate without IPTG induction

Lane 2: The cell lysate with IPTG induction

Lane 3: The purified hG-CSF

Figure 4 represents the in-vitro activity assay of hG-CSF from the recombinant.

DETAILED DESCRIPTION OF THE INVENTION

The plasmid, pYHM-G-CSF, was derived from an expression vector, pET 22b(+) in E. coli. The cDNA of hG-CSF was introduced in the multi-cloning site of pET 22b(+). The purpose of the plasmid was to increase the expression rate of hG-CSF by T7/lac promoter with IPTG and to release hG-CSF into the culture media after removing signal peptide.

But, the present inventors obtained the hG-CSF without removing the signal peptide in the bacteria. The fusion protein was highly accumulated (1.7g/L culture broth) in the bacteria and has the biological activity of hG-CSF even though an additional 20 amino acid sequence on N-terminus. This material is a new protein and is named as human granulocyte colony-stimulating factor (hG-CSF). hG-CSF can be used for the clinical and research purpose and the primary material of hG-CSF production.

The plasmid, pYHM-G-CSF, provided the high expression of hG-CSF, which was activated by oxidative refolding method (KP No. 95-2424).

Example 1: Synthesis of cDNA for hG-CSF

cDNA fragments for hG-CSF were synthesized as Figure 1 and were recovered by Mupid-2 (Cosmo Bio Co.)(US patent No. 4,833,127). The cDNA fragments were annealed and ligated as the order of oligomers in Figure 1 (The condition of annealing and ligation: Maniatis et al., Molecular cloning: A Laboratory Manual, Cold Spring Harber Laboratory, 1982). The sites for NcoI and BamHI were introduced at 5′ and 3′ terminus of the produced cDNA, respectively.

Example 2: Construction of plasmid, pYHM-G-CSF

pET 22b(+)(Novagen Co.) was digested with NcoI and BamHI. cDNA for hG-CSF which has NcoI and BamHI sticky ends was inserted and ligated in the pET 22b(+), resulting in 5.9 kb of a plasmid, pYHM-G-CSF.

Recombinant bacteria were derived from E. coli BL21(DE3) by the introduction of pYHM-G-CSF. The recombinant bacteria were registered as registration number KCTC 0477BP to the Gene bank in the Korea Research Institute of Bioscience and Biotechnology (KRIBB), KIST on February 21, 1997. The recombinant was cultured in LB media with 0.1 mg/mL of ampicillin for overnight.

Example 3: Expression

E. coli (BL21/pYHM-G-GSF) was cultured in 100 mL of LB media (0.01 g Bacto trypton, 0.005 g Bacto yeast extract and 0.01 g NaOH per mL, pH 7.5 titrating with pH 7.5) for 1.5 hour at 37°C to reach 0.3 of absorbance at 600 nm. E. coli was cultured for another 7 hours after the addition of 0.5 mM final concentration of IPTG.

SDS-PAGE was performed on 12 % acrylamide with the culture media and the 19 kd of the protein was identified. The hG-CSF contained 40-45 % of the total E.coli protein.

Example 4: Purification

E. coli (BL21/pYHM-G-GSF) was cultured in 100 ml of LB media including 0.1 mg/mL ampicillin for 4 hour at 37°C. E. coli was cultured for another 9 hours after the addition of 0.25 mM final concentration of IPTG. The hG-CSF protein was produced and the amount was 1.7g/L. The cells were centrifuged and suspended with 30mL of a solution (50 mM Tris-Cl, pH 7.5, 1 mM EDTA).

After breaking down with sonicator (Branson, Sonifier 450) it was centrifuged at 4°C, 5,000 rpm. The pellet was collected, washed with 4 M urea solution (4 M urea, 0.5% Tween 20, 1 mM EDTA in 50 mM Tris-Cl, pH 8.0) twice, and dissolved with 8 M urea buffer (8 M urea, 10 mM DTT, 1 mM

EDTA in 40 mM Tris-Cl, pH 8.0).

The solution was purified with DEAE-sepharose CL 6B column (4.5 X 15 cm). The effluent was collected, concentrated by ultrafiltration system (Amicon Co.), and purified with Sephacryl S-200HR column (2 X 130 cm). The protein was analyzed with 12% SDS-PAGE and was stained with coomassie brilliant blue solution. The gel was dried. Figure 3 represents the result and purity of the protein was 99.5%.

SDS-PAGE gel was equilibrated with a solution of 190 mM Glycine, 25 mM Tris-Cl, pH 8.3 for 30 min. It was transferred onto nitrocellulose membrane which was treated with 5% skim milk. The treated membrane was reacted with rabbit polyclonal antibody (Genzyme Co.) (1:200 dilution) for 1 hour. Then, the membrane was reacted with a solution (1:1000 dilution) of goat anti-rabbit IgG-alkaline phosphatase conjugate for 1 hour. Color development was performed with a solution of NBT/BCIP substrate. Molecular weight of the purified protein was about 19,000.

Example 5: Determination of N-terminal sequence

The N-terminal sequence of the purified protein was determined by automatic peptide sequencer (Miligen/Biosearch Co., Model 6600 prosequencer).

The peptide from signal sequence and first 10 amino acid from hG-CSF were determined, which are:

Signal peptide: Lys Tyr Leu Leu Pro Thr Ala Ala Ala Gly Leu Leu Leu Leu Ala Ala Gln Pro Ala

First 10 amino acid from hG-CSF: Met Thr Pro Leu Gly Pro Ala Ser Ser Leu

Therefore, we named the peptide as human granulocyte colony-stimulating factor (hG-CSF).

Example 6: Oxidative refolding

The protein was dissolved at 0.5 mg/mL and room temperature in the a solution of 2 mM reduced glutathion, phosphate buffer, pH 7.5 for 3 hours.

The yield of hG-CSF was calculated by peak area at 220nm, using HPLC method with YMC-Pack Protein-RP column. HPLC analysis were performed at a rate of 0.8 mL/min with acetonitrile and water containing 0.1% trifluoroacetic acid. The buffer condition was a linear gradient of acetonitrile-water ratio from 35:65 to 65:35 for 45 min. Active, unfolded, and mis-matched forms were detected at 23, 24, and 27.3 min, respectively. The yield of active form was above 60%.

Example 7: The measurement of bioactivity

A cell line (M-NFS-60) from leukemia was cultured with RPMI 1640 media (additionally, 2 mM glutamine, 0.05 mM 2-mercaptoethanol, G-CSF 10 ng/mL) for 2-3 generation. The cells were collected by centrifugation, washed with a buffer for three times, and diluted to 10^5 cells/mL.

The cells were divided into 96-well plate with 50 uL, individually. The purified protein were diluted to a series of concentration, 0.1, 1.0, 10.0 ng/mL and were added to 100 uL/well. The plates were incubated with 5% carbon dioxide at 37°C for 22 hours. After adding ^{3}H-thymidine (Amersham Co.) of 37 KBg/50uL/well, the plates were further incubated for 6 hours, washed with a buffer three times, and added 100 uL of 0.1 M NaOH. The collected cells were transferred to scintillation counter vials. After adding 2.5 mL cocktail solution (Amersham Co.) radioactivity of the vials were determined. Grasin from Kirin Brewry Co. was used as a standard. The experiment was triplet. Figure 4 represents the results.

Activity of the purified protein (hG-CSF) was similar to Grasin from Kirin Brewry Co. from Figure 4.

WHAT IS CLAIMED IS:

1. Human granulocyte colony-stimulating factor containing a peptide of Lys Tyr Leu Leu Pro Thr Ala Ala Ala Gly Leu Leu Leu Leu Ala Ala Gln Pro Ala on N-terminus of hG-CSF.

2. The protein of claim 1, which is accumulated above 1.7 g/L in the transformed bacteria containing cDNA for the peptide of claim 1.

3. The recombinant plasmid, pYHM-G-CSF, which contains cDNA encoding the peptide of claim 1

4. The Escherichia coli BL21(pYHM-G-CSF) (KCTC 0477BP), which are transformed with the recombinant plasmid, pYHM-G-CSF.

5. The production method of the peptide of claim 1, using the plasmid of claim 3. The peptide of claim 1 is expressed from the culture of the bacteria of claim 4. The cells are collected by centrifugation, disrupted by sonication. The precipitate is dissolved with urea. The solution is passed through DEAE-Sepharose CL 6B and Sephacryl S-200 HR columns, and concentrated by

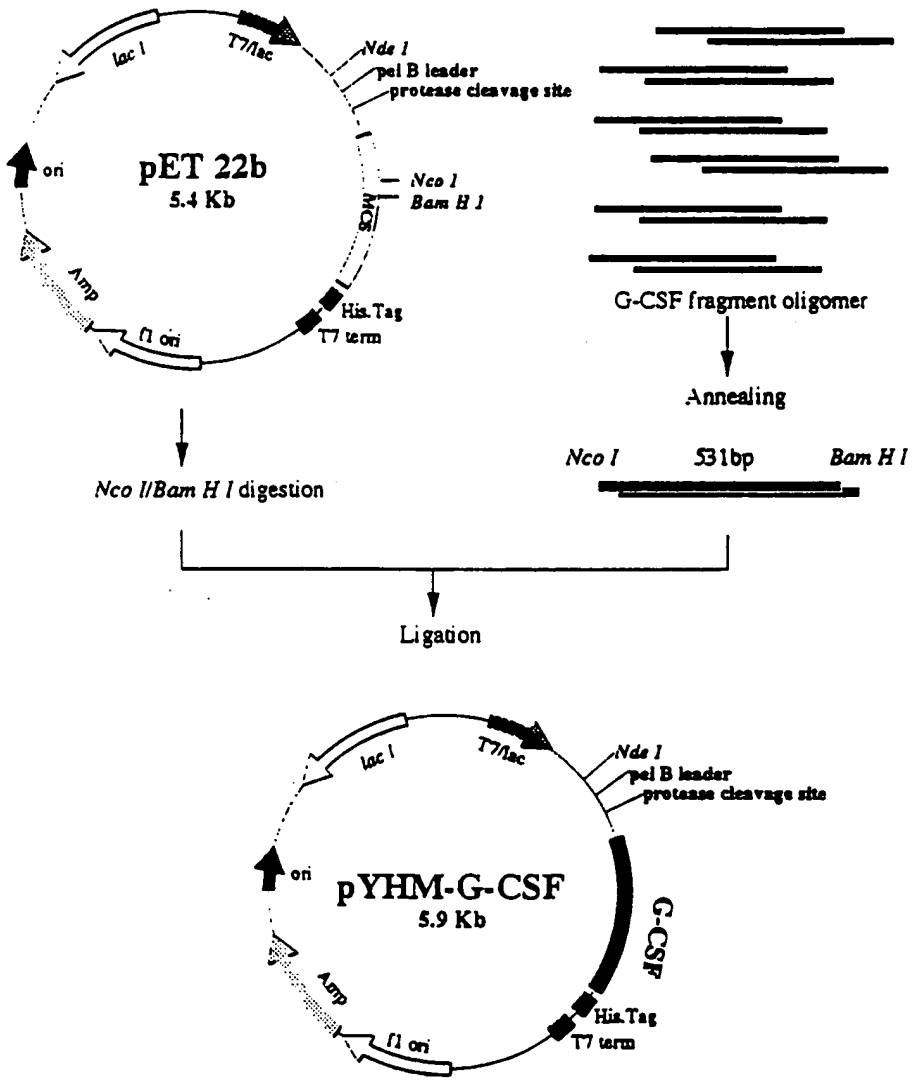
ultrafiltration. The p ptide of claim 1 is refolded for activation.

FIG. 1

[DIAGRAM]

Oligo 1	Sense	5'	CATGACTCCACTGGGTCCTGCCAGCTCCCTGCCGCAGAGTC 3'
	Antisense		3' TGAGGTGACCCAGGACGGTCGAGGGACGGCGTCTCGAAGGACGAG 5'
Oligo 2	Sense	5'	TCCTGCTCAAGTGCTTAGAGCAAGTGAGGAAGATCCAGGGC 3'
	Antisense		3' TTCACGAATCTCGTTCACTCCTTCTAGGTCCCGCTACCGCG 5'
Oligo 3	Sense	5'	GATGGCGCAGCGCTCCAGGAGAAGCTGTGTGCCACCTACAA 3'
	Antisense		3' TCGCGAGGTCCTCTTCGACACACGGTGGATGTTCGACACGG 5'
Oligo 4	Sense	5'	GCTGTGCCACCCGGAGGAGCTGGTGCTGCTCGGACACTCTC 3'
	Antisense		3' TGGGCCTCCTCGACCACGACGAGCCTGTGAGAGACCCGTAG 5'
Oligo 5	Sense	5'	TGGGCATCCCGTGGGCTCCGCTGAGCAGCTGCCCGAGCCAG 3'
	Antisense		3' GGCACCCGAGGCGACTCGTCGACGGGCTCGGTCCGGGACGT 5'
Oligo 6	Sense	5'	GCCCTGCAGCTGGCAGGCTGCTTGAGCCAACTCCATAGCGG 3'
	antisense		3' CGACCGTCCGACGAACTCGGTTGAGGTATCGCCGGAAAAGG 5'
Oligo 7	sense	5'	CCTTTTCCTCTACCAGGGTCTCCTGCAGGCCCTGGAAGGGA 3'
	antisense		3' AGATGGTCCCAGAGGACGTCCGGGACCTTCCCTAGAGAGGC 5'
Oligo 8	sense	5'	TCTCTCCGGAGTTGGGTCCCACCTTGGACACACTGCAGCTG 3'
	antisense		3' CTCAACCCAGGGTGGAACCTGTGTGACGTGGACCTGCAGCG 5'
Oligo 9	sense	5'	GACGTCGCCGACTTTGCCACCACCATCTGGCAGCAGATGGA 3'
	antisense		3' GCTGAAACGGTGGTGGTAGACCGTCGTCTACCTTCTTGACC 5'
Oligo 10	sense	5'	AGAACTGGGAATGGCTCCTGCTCTGCAGCCGACCCAGGGTG 3'
	antisense		3' CTTACCGAGGACGAGACGTCGGCTGGGTCCCACGGTACGGC 5'
Oligo 11	sense	5'	CCATGCCGGCCTTCGCCTCTGCTTTCCAGGCCGGGCAGGA 3'
	antisense		3' CGGAAGCGGAGACGAAAGGTCGCGGCCCGTCCTCCACAGGA 5'
Oligo 12	sense	5'	GGTGTCCTGGTTGCCTCCCATCTGCAGAGCTTCCTGGAGGT 3'
	antisense		3' CCAACGGAGGGTAGACGTCTCGAAGGACCTCCACAGCATGG 5'
Oligo 13	sense	5'	GTCGTACCGCGTTCTACGCCACCTTGCCCAGCCCTAATAG 3'
	antisense		3' CGCAAGATGCGGTGGAACGGGTCGGGATTATCCTAG 5'

FIG. 2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5.4 Kb
lac I
T7/lac
Nde I
pel B leader
protease cleavage site
Nco I
Bam H I
MCS
His.Tag
T7 term
f1 ori
Amp
ori
G-CSF fragment oligomer
Annealing
Nco I
531bp
Bam H I
Nco I/Bam H I digestion
Ligation
pYHM-G-CSF
5.9 Kb
G-CSF

FIG. 3

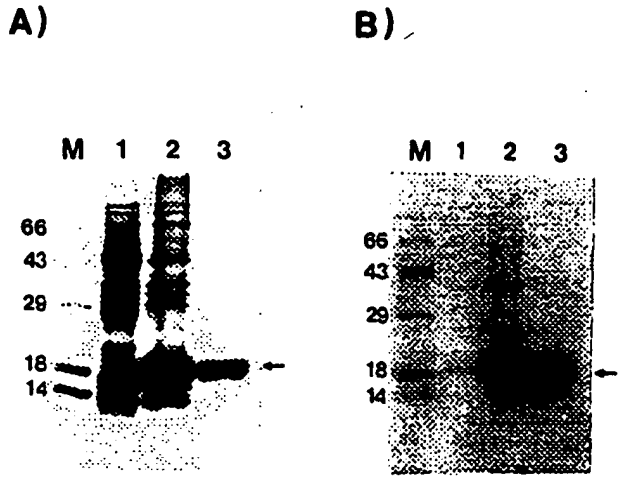
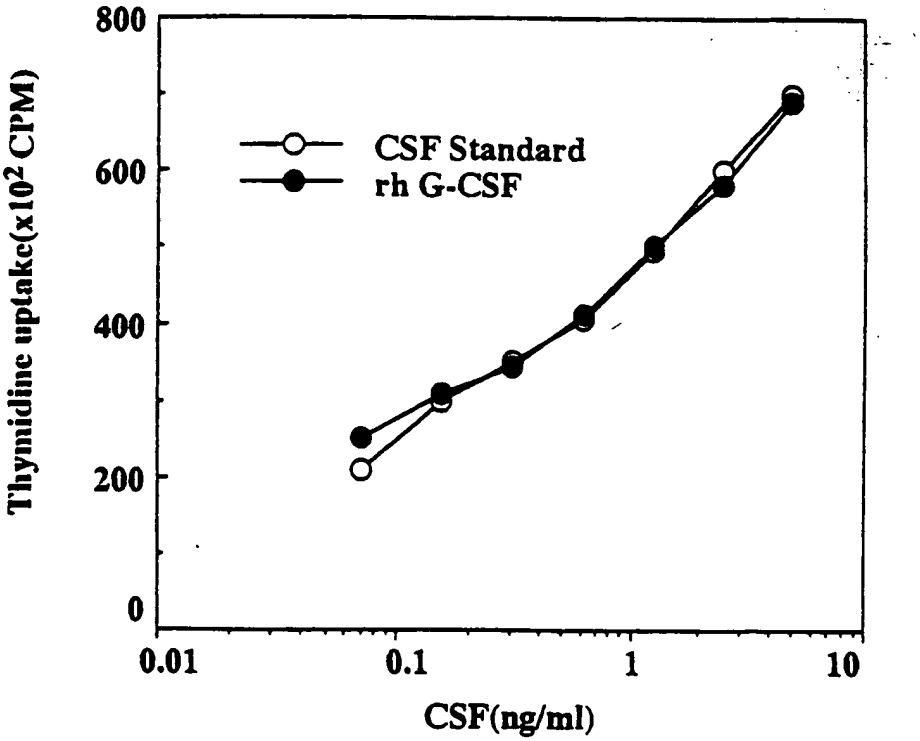


FIG. 4


800
600
400
200
0
Thymidine uptake(x10² CPM)
CSF Standard
rh G-CSF
0.01
0.1
1
10
CSF(ng/ml)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/KR 98/00125

A. CLASSIFICATION OF SUBJECT MATTER

IPC[6]: C 12 N 15/27, 1/21 // (C 12 N 1/21; C 12 R 1:19)

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Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

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C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	EP 0 243 153 A2 (IMMUNEX CORPORATION) 28 October 1987 (28.10.87), claims; fig.1.	1-5
A	EP 0 344 796 A2 (CHUGAI SEIYAKU KABUSHIKI KAISHA) 06 December 1989 (06.12.89), claims 1-4.	1

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Date of the actual completion of the international search	Date of mailing of the international search report
20 August 1998 (20.08.98)	26 August 1998 (26.08.98)

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Information on patent family members

International application No.

PCT/KR 98/00125

In Recherchenbericht angeführtes Patentdokument Patent document cited in search report Document de brevet cité dans le rapport de recherche	Datum der Veröffentlichung Publication date Date de publication	Mitglied(er) der Patentfamilie Patent family member(s) Membre(s) de la famille de brevets	Datum der Veröffentlichung Publication date Date de publication
EP A2 243153	28-10-87	AU A1 71844/87	29-10-87
		AU B2 601727	20-09-90
		DK A0 2031/87	21-04-87
		DK A 2031/87	23-10-87
		EP A3 243153	13-01-88
		JP A2 63000299	05-01-88
		ZA A 8702705	30-12-87
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